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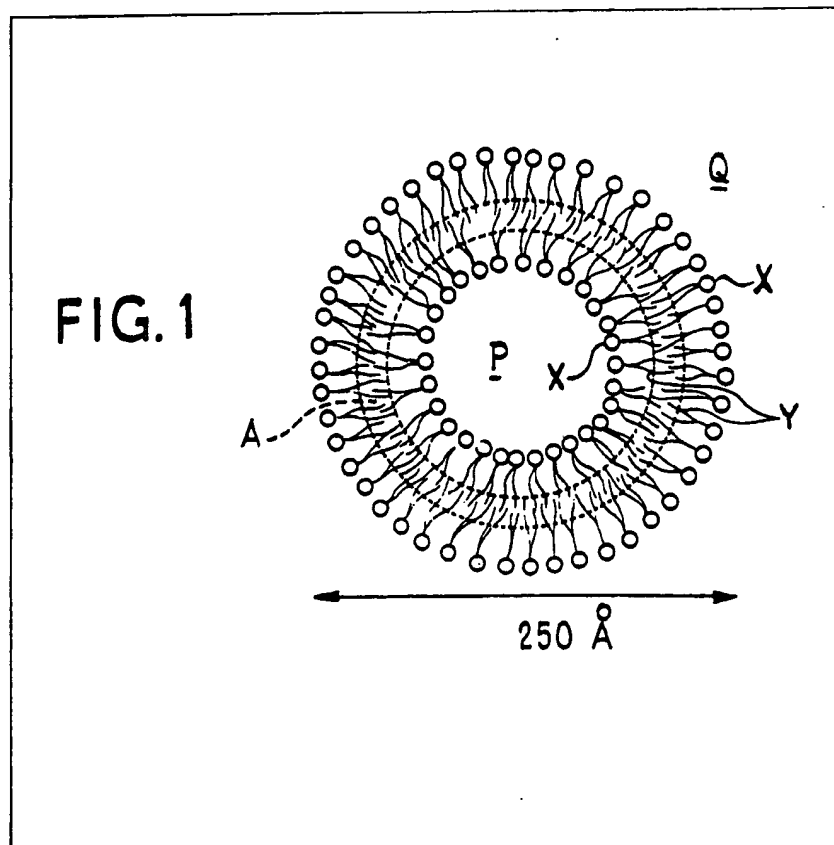
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(54) Liposomes containing physiologically active substances

(57) Membrane walls (X, Y) of liposomes further comprise an oily substance (A) such as a mineral oil, wax or triglyceride. These liposomes can be used to deliver a physiologically active substance (P) to a tissue within a living body over a period of time.



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FIG. 1

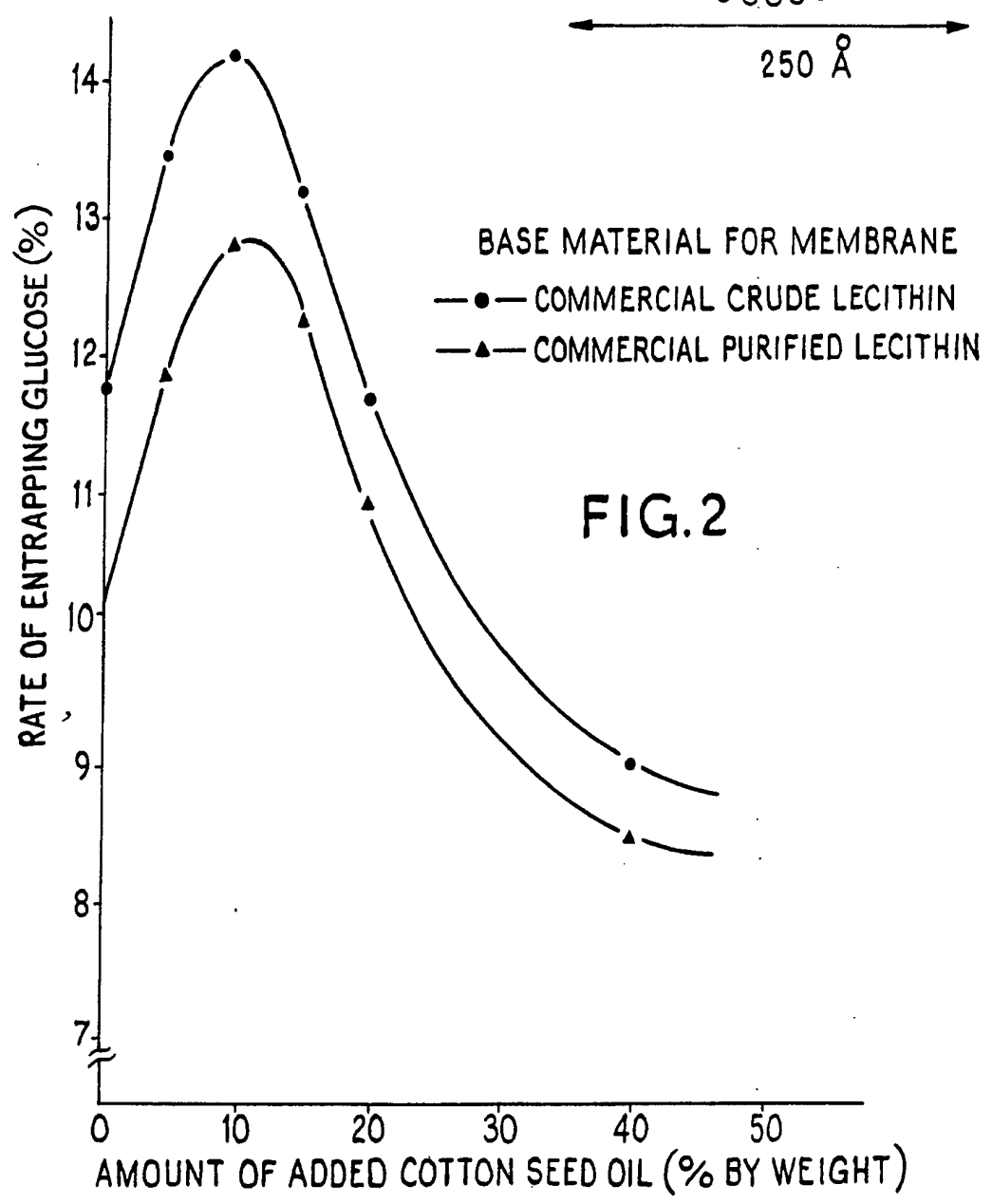
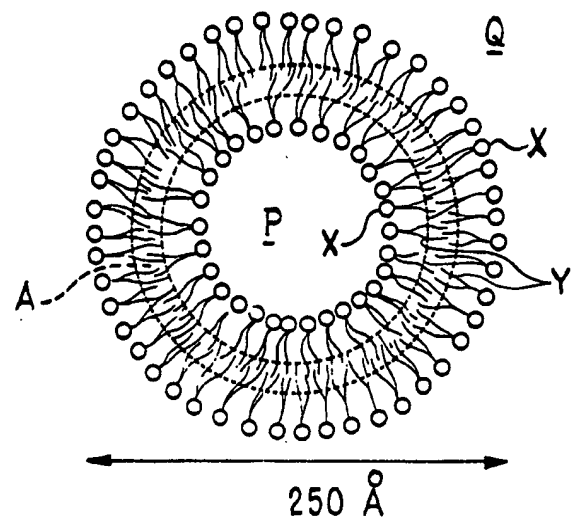
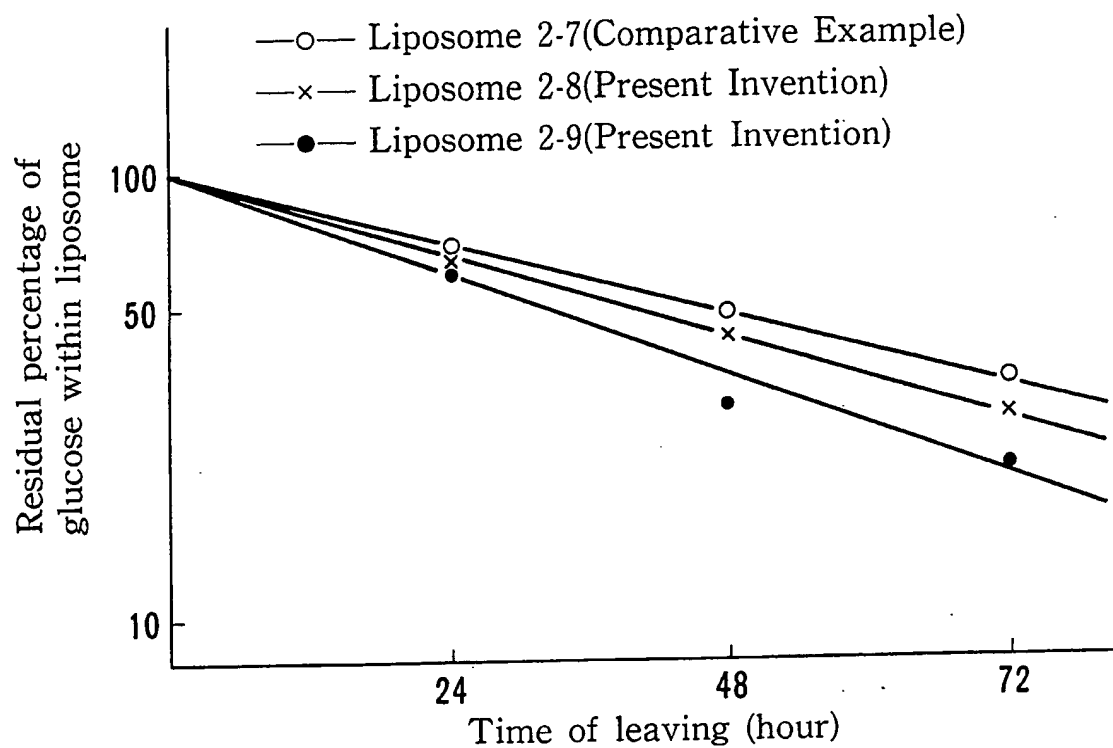
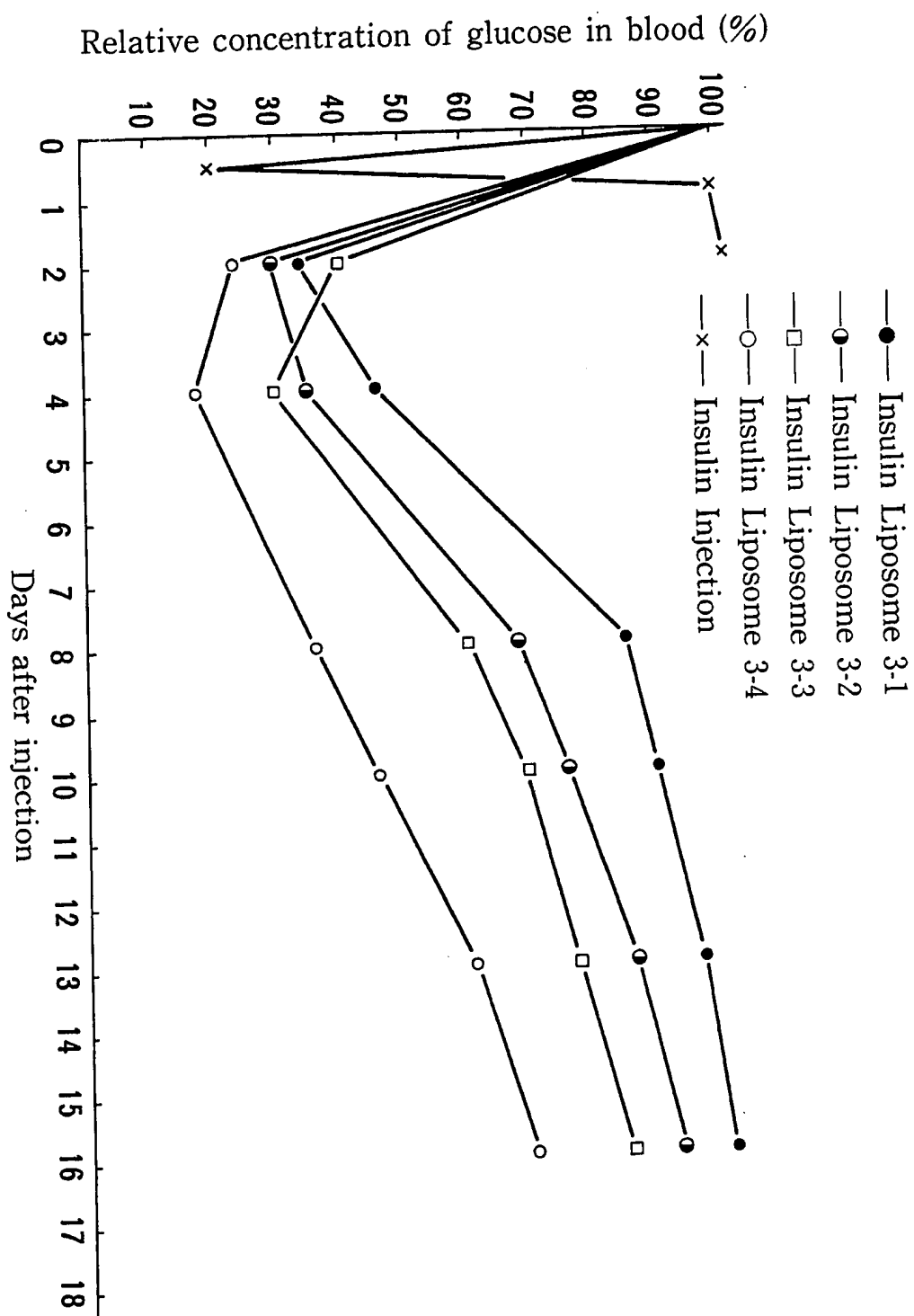


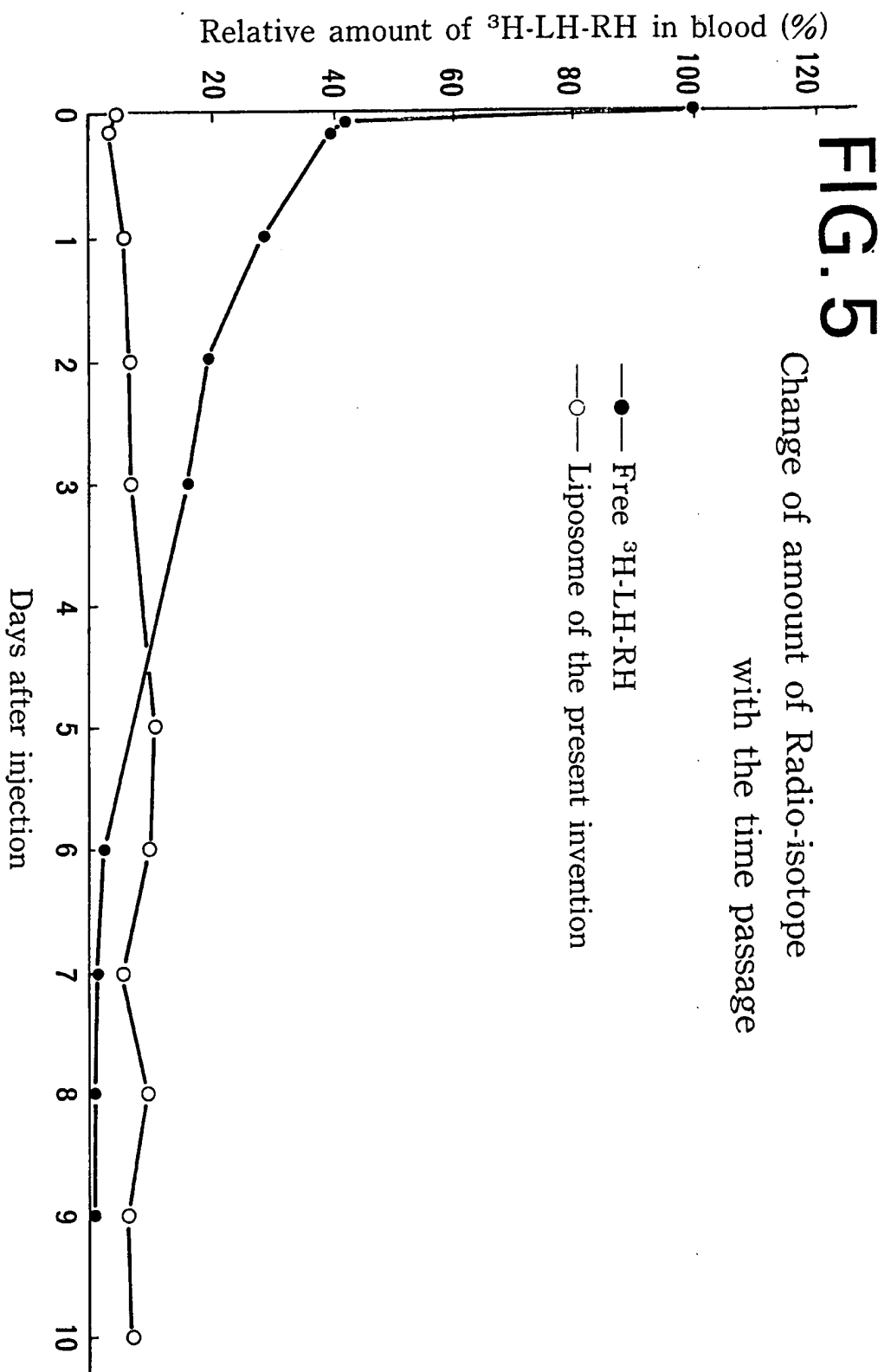
FIG. 2

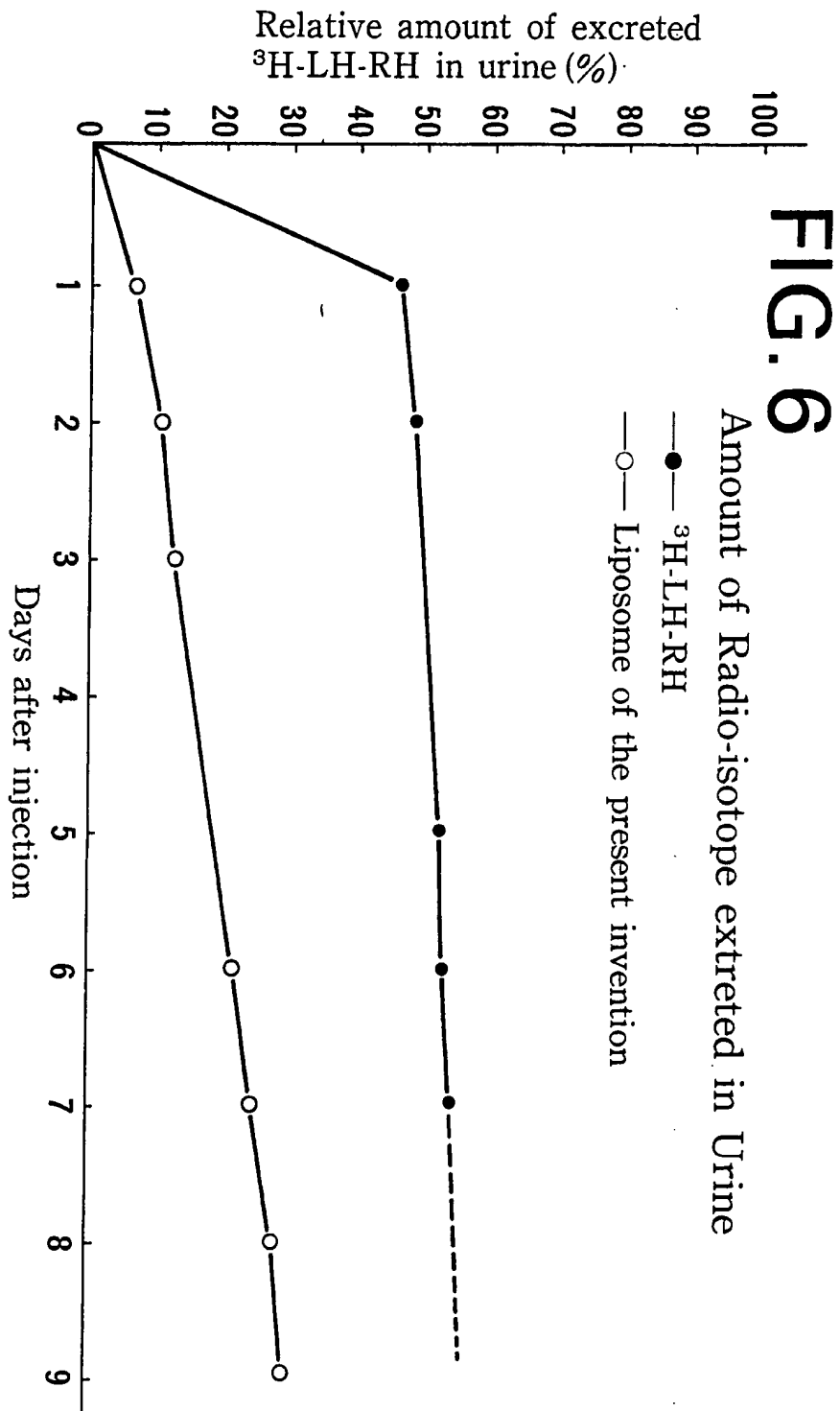
**FIG. 3** Permeability of Glucose

**FIG. 4** Persistency of Active Substance (Insulin)  
within the living body



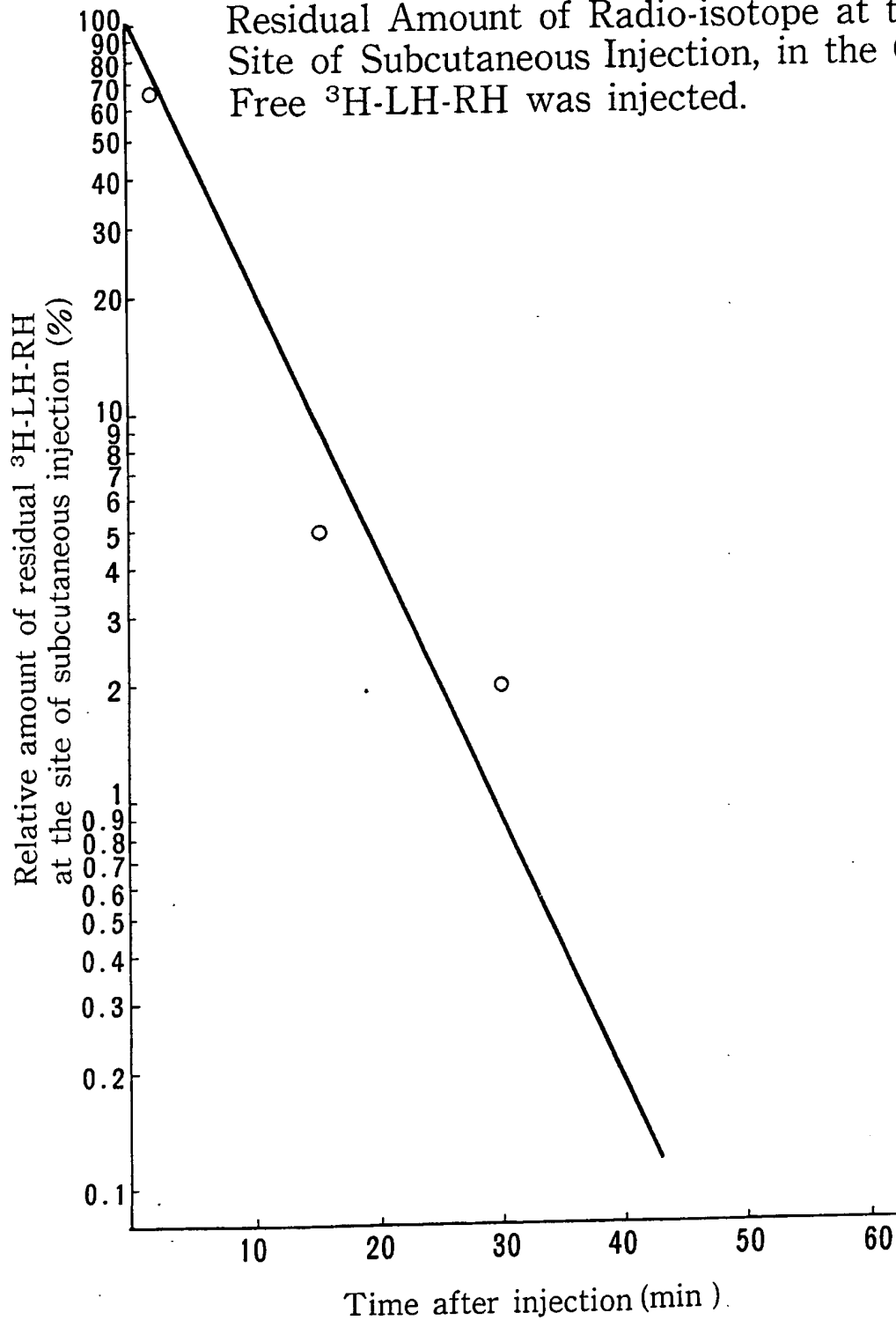
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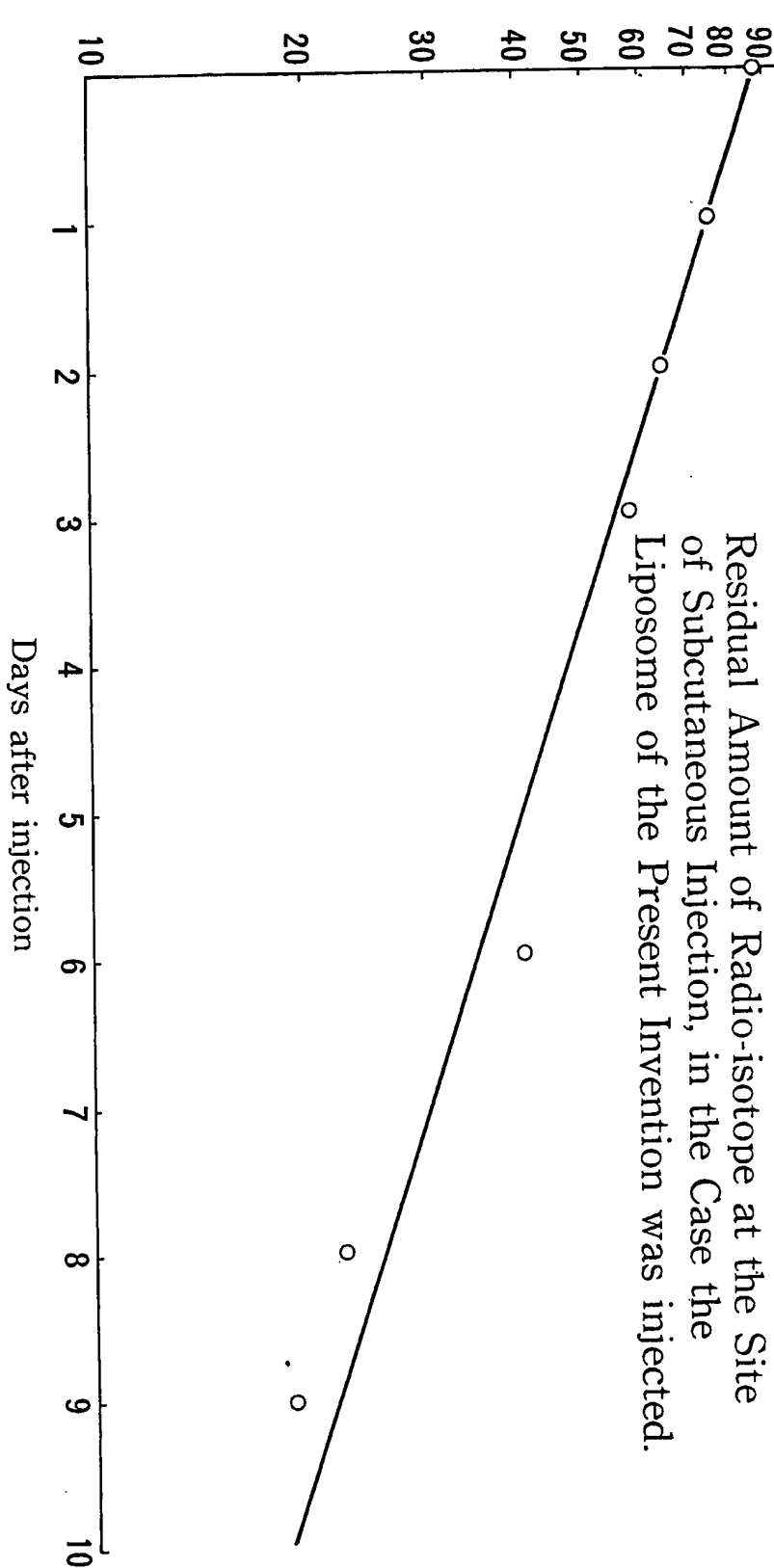


**FIG. 7**

Residual Amount of Radio-isotope at the Site of Subcutaneous Injection, in the Case Free  $^3\text{H}$ -LH-RH was injected.



Relative amount of residual  $^3\text{H}$ -LH-RH  
at the site of subcutaneous injection (%)

**FIG. 8**



## SPECIFICATION

## Liposome containing a physiologically active substance

- 5 The present invention relates to liposomes including an active substance, particularly a physiologically active substance. 5
- Hitherto, where a physiologically active substance such as a medicine is directly administered to a living body, there have been many instances of (1) immunological problems caused by the proliferation within the body of antibodies to the medicine in question, (2) side effects resulting from the uptake of the medicine by the tissues other than the intended target of the medicine, (3) problems caused by the inability of the medicine to reach the target tissue, and (4) problems caused by the inability of the medicine to maintain its activity because of its degradation, inactivation, etc. by enzymes within the body. These difficulties could be resolved by administering the physiologically active substance loaded onto a carrier which is able to transfer the active substance directly to the target tissue within the living body while protecting the active substance.
- 10 In an effort to achieve this ideal, Japanese Patent Application Laying Open No. 118826/74 (DE-OS-2249 552) has recently proposed the use of liposomes comprising vesicles having closed lamellar structure (micelle) and containing a physiologically active substance dissolved in an aqueous solution. The vesicles are composed of at least one bimolecular layer as a membrane wall formed by a compound represented by the general formula X-Y, wherein X represents a polar, hydrophilic group such as phosphate, carboxyl, amino, hydroxyl or choline and Y represents a non-polar, hydrophobic group such as alkyl, alkenyl or alkynyl. Examples of such compounds include purified phospholipids such as lecithin, phosphatidylethanolamine and phosphatidylserine. Since the membrane wall which forms the liposome protects the active substance in the aqueous solution within the vesicle even under severe conditions, for instance, those encountered in the gastrointestinal tract, the activity of the active substance is not lost even when the liposome is administered orally. The addition, since the permeability of the liposome to the tissue of a living body is dependent upon the liposome's particle size (diameter), it is possible to raise this permeability by adjusting the liposome diameter. Accordingly, liposome has been considered as a possible means of selectively supplying a physiologically active substance to a particular tissue within a living body.
- 20 However, the wall membrane of the liposome described above and which consists of pure phospholipid lacks pliability and has insufficient strength. In addition, owing to the speed with which the liposome releases the physiologically active substance, it is not necessarily satisfactory as regards slowly releasing the active substance within the living body over a period of time, that is its so-called slow-releasing property. In particular the speed with which such a liposome releases the active substance is markedly increased at temperatures higher than the transition temperature of the wall membrane of the liposome.
- 25 In order to improve the strength of the wall membrane steroid lipids such as cholesterol have been mixed with phospholipids. Although the strength of the wall membrane is somewhat improved by this technique, the slow-releasing property of the liposome is not much improved.
- It has now been discovered that membrane walls of liposomes which have been formed from a phospholipid containing molecules of an oily substance, such as "crude lecithin", can have a higher pliability and a better slow-releasing property than membrane walls of known liposomes formed by using purified phospholipid. Accordingly, the present invention provides a liposome comprising a vesicle, the membrane wall of which comprises a bimolecular layer of phospholipid incorporating at least one oily substance, said at least one oily substance being present in an amount of 3 to 20% by weight of said phospholipid, and at least one physiologically active substance contained within the vesicle.
- 30 The present invention also provides a composition for forming such a liposome, which composition comprises a phospholipid, at least one oily substance and at least one physiologically active substance.
- In the description which follows, reference will be made to the accompanying drawings in which:
- Figure 1* is a schematic illustration of a liposome in accordance with the present invention;
- Figure 2* shows, for a liposome according to the present invention containing glucose in the aqueous solution within the vesicle of the liposome, the relationship between the amount of oily substance contained in the membrane material forming the liposome, and the percentage of the amount of glucose trapped within the vesicle based on the total amount of glucose used to form the liposome (expressed hereinafter as the rate of entrapment);
- Figure 3* shows a comparison of the permeability of the glucose-loaded liposomes of Specimens 2-8 and 2-9 of Example 2 in accordance with the present invention and of the liposomes of Comparative Specimen 2-7 of Example 2;
- Figure 4* shows a comparison of the persistency of the hypoglycemic action of insulin in a living body, which is supplied by the liposome of the present invention containing insulin therewithin to that by the liposome of Comparative Example also containing insulin therewithin.
- Figures 5 to 8* show the slow-releasing property to an active substances included within the respective liposomes of the present invention.
- Among them, *Figure 5* shows the change of the amount of radio-isotope in blood with the passage of time after the subcutaneous injection of the liposome prepared by using the above-mentioned substance, however, radio-labelled, as the active substance, and *Figure 6* shows the change of the amount of radio-isotope in urine with the passage of time after the same treatment as above. *Figure 7* shows the

change of the residual amount of radio-isotope, at the site where the free radio-labelled active substance was directly injected subcutaneously as it is, with the passage of time, and Figure 8 shows the change of the residual amount of radio-isotope, at the site where the liposome including the radio-labelled active substance within its vesicle was injected subcutaneously, with the passage of time.

- 5 The characteristic feature of the present invention is, on the formation of a liposome including an active substance within its vesicle surrounded by a micellar membraneous layer, the use of a material comprising micellar membraneous layer of phospholipid, in which molecules of a fatty substance are present as the wall membrane of the above-mentioned liposome. 5

- 10 Another characteristic feature of the liposome, including an active substance, formed by using the above-mentioned material according to the present invention is its W/O/W-type complex emulsified state as shown in Figure 1 and its excellent property of slowly releasing the above-mentioned active substance included within its vesicle to the outside when taken into a living body. In Figure 1, X represents a hydrophilic group, Y a hydrophobic group, P a vesicle containing an aqueous solution, A molecules of fatty substance and Q an aqueous solution outside of liposome, respectively. 10

- 15 The material for forming the liposome of the present invention, as has been described above, comprises phospholipid with which molecules of a fatty substance are mixed or to which they are bonded. The phospholipid used herein is not particularly limited provided it has been used as the material for a membraneous layer of the conventional liposome, for instance, a single compound of lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserin, phosphatidylinositol, sphingomyelin, cardiolipin, etc. or its mixture, and according to the necessity, a sterol compound such as cholesterol may be contained. 15 20

The fatty substance present in the above-mentioned phospholipid is triglyceride, wax or mineral oil or the mixture thereof, for instance, those selected from vegetable oil such as soy-bean oil, cotton seed oil and sesame oil, and also from mineral oils of coil-origin or petroleum-origin.

- 25 Particularly preferable is the single use of crude lecithin or the use of a mixture of crude lecithin and other oily substance mentioned above. 25

- The term "crude lecithin" herein used means a fraction which eluates with chloroform or a mixture of chloroform and methanol in a ratio of 100 : 1 to 3 : 2 when a component rich in phospholipid derived from substance such as yolk and soybean oil is fractioned by column chromatography using alumina as a column material, the fraction consisting of 97 to 80% by weight of pure lecithin and 3 to 20% by weight of oily substance, for instance, triglyceride and carotenoid. 30

- In cases where the liposome of the present invention is prepared by using the above-mentioned crude lecithin as a material for membraneous layer, the content of the oily substance in crude lecithin is adjusted so that the content of the fatty substance in the thus formed wall membrane becomes to 3 to 20% by weight, preferably 5 to 15% by weight. In addition, care should be taken to keep the content of the fatty substance in the above-mentioned wall membrane not over 20% by weight or the formation of the wall membrane is impaired to have reduced yield of the liposome. On the other hand, in the case where the content of the fatty substance is less than 3% by weight, the above-mentioned object of the present invention cannot be attained. 35

- 40 By the way, on the formation of the liposome according to the present invention, sterols such as cholesterol and ergosterol and a substance capable of changing the electrically loaded state of the surface of the liposome, for instance, phosphatidic acid, dicetyl phosphate or ganglioside of bovine brain for giving negative charge, or stearylamine for giving positive charge may be admixed with the material for membraneous layer as the third component. The amount of addition of the third component may be determined adequately according to the property of the phospholipid suitably, and usually is 0 to 10% by weight of the material for membraneous layer. 45

- In the case of forming the liposome of the present invention while using a mixture of the above-mentioned phospholipid and the oily substance as a material for membraneous layer, the conventional method for preparing a liposome is possibly applied. For instance, a method in which a thin film is formed from the above-mentioned material and after bringing the thus formed membrane into contact with a continuous phase containing an active substance to form a dispersion by agitation, a supersonic vibration is applied on the dispersed system, a method in which after mixing a solution of the above-mentioned material for membraneous layer in a solvent insoluble in water with an aqueous solution containing the above-mentioned (water-soluble) active substance, the mixture is subjected to a supersonic treatment for forming a precursor of liposome and then the solution containing the precursor is subjected to ultracentrifugal treatment in the co-present of an aqueous medium, or a method in which after coating the surface of glass-beads and the like with the above-mentioned material for membraneous layer, the coated beads are mixed with an aqueous solution containing the above-mentioned active substance to disperse the coated beads into the solution is applicable. 50 55

- 60 The amount of the above-mentioned material for membraneous layer on forming the above-mentioned liposome is 1 to 500 mg/ml of the liquid in which the liposome is suspended. 60

- The membraneous layer of the liposome obtained by the above-mentioned method is formed as a result of mutual action between the hydrophobic group possessed by the phospholipid in the material for membraneous layer which forms the liposome and the molecules of oily substance which are present in the material for membrane layer. Accordingly, the morphological state of the liposome of the present invention 65

is to be said quite different substantially from the state of the conventional liposome comprising the micelle of purified phospholipid.

In addition, the oily substance as a constituent of the wall membrane of the liposome according to the present invention, on the formation of the liposome from the material for membraneous layer, brings about the improvement of the liposome-yield, and after forming the liposome, brings about the easiness in the process of separating the liposome, the uniformity of the particle size of the liposome and the improvement of pliability, the strength of the wall membrane of the liposome and the slow-releasing property of the active substance included in the vesicle of the liposome when administered within the living body.

The active substance, particularly the physiologically active substance used on the formation of the liposome of the invention is a medicine of which the side effect is to be reduced or a medicine of which the persistency within the living body is to be improved, and is exemplified by peptide hormones such as insulin, oxytocin, vasopressin, adrenal-cortex stimulating hormone (ACTH), luteinizing hormone-releasing hormone (LH-RH), cartitinin, somatostatin, steroid-hormones such as progesterone, follicular hormone, adrenal cortex hormone and other hormones such as prostaglandin, adenosine-3', 5'-cyclicmonophosphate, anti-tumor agents such as chlorambutyl, streptozocin, methothrexate, 5-fluorouracil, sitosin arabinoside, mitomycin C, breomycin, polysaccharide derivative, antibiotics such as penicillin, cepharospolin, streptomycin, enzymatic preparations such as aminoglucosidase, invertase, etc.

The liposome according to the present invention comprises particles of 0.01 to 10 microns in average diameter with a narrow range of distributions of particle diameter, and those particles of 0.5 to 5 microns in average diameter are obtainable easily with a uniform diameter. The particles of liposome of the present invention are excellent in pliability, and they can be concentrated by a low speed-centrifugal treatment at 2,000 to 4,000 r.p.m. Moreover, the re-dispersion after concentration can be carried out only by a short time-shaking without necessitating an ultrasonic treatment to be recovered to the original state.

The liposome according to the present invention, as is understood from its specific properties described above, is excellent in its capability of maintaining the active substance included within its vesicle compared to the conventional liposome of the type of pure phospholipid micelle, and also its slow-releasing property to the active substance included therewithin is favorable. Accordingly, the liposome of the present invention is particularly suitable as a protecting agent for an unstable medicine within the living body, and also for a medicine which causes side effects by an inevitable excessive administration. By these reasons, the liposome according to the present invention is possibly utilisable as a medicine.

For instance, in the case where the liposome of the present invention is applied as an insuline injection, an intra-muscular administration of the liposome of the present invention once two to seven days at a dose of 0.2 - 20 mg as insulin to an adult gives the same effect as that of the direct intramuscular administration of free insulin three times a day at a single dose of one third of 0.1 to 4.0 mg.

In cases where the liposome of the present invention is utilized as a medicine, its administration is possible via several routes such as percutaneous, subcutaneous, intramuscular, intraperitoneal, intravenous, intrarectal and topical, preferably by subcutaneous or topical administration. The amount of administration depends naturally to the method and the route of administration, the kind of active substance and the extent of treatment, however, usually it is 0.1 to 1 times as large as the amount of the active substance directly administered per day, and further the interval of administrations can be extended.

In addition, the liposome of the present invention is possibly administered as an injection after dispersing the liposome into an aqueous physiological saline solution. Particularly, the liposome of the present invention containing a peptidic physiologically active substance including peptide hormones exhibits a remarkable effect when applied as a subcutaneous or intramuscular injection. Since peptidic physiologically active substances are generally degraded promptly in the living body, it is necessary to carry out frequent administration (injection) for the maintenance of the effect of the substance resulting not only in causing the heavier load on the patient but also causing the large fluctuation of the concentration of the active substance in blood. These situations are apt to reduce the effect of administration of the active substance and to cause side effects of the active substance.

Whereas, the liposome of the present invention, as are shown later in Examples, shows an excellent slow release of the included active substance on subcutaneous or intramuscular injection, and accordingly, the times of administration are possibly reduced materially with a maintenance of the uniform concentration of the peptidic physiologically active substance in blood, and accordingly, the effect of the active substance is fully exhibited with the suppression of its side effects.

According to the acute toxicity test of the above-mentioned materials for forming the above-mentioned liposome of the present invention using rats via two routes of subcutaneous and intravenous injections, no toxic sign was observed on the treated rats until the dose rate reached 1,000 mg/kg concerning every material, the fact showing that the liposome of the present invention is safely applicable as a medicine from the viewpoint of the wall membrane of the liposome.

The followings are the concrete explanation of the present invention while referring to Examples.

#### Example 1:

A solution containing 100 mg of a commercial crude yolk lecithin (manufactured by Merck Company), 11.6 mg of cholesterol and 2.7 mg of stearylamine in 10 ml of chloroform was placed in a 25-ml round-bottomed flask set on a rotatory evaporator, and by distilling chloroform from the solution while rotating the

evaporator at a temperature of 38°C under reduced pressure, a film was formed on the inner wall of the flask. Then, one milliliter of an aqueous 1% by weight solution of adenosine 3', 5'-cyclic-monophosphate (hereinafter abbreviated as C-AMP) was added to the flask, and by shaking the flask for 30 minutes the film was exfoliated from the inner wall of the flask and the film was dispersed into the solution. By treating the dispersion with a  
 5 supersonic treating machine (manufactured by Nippon Seiki Company, Model NS 200-2) for 20 min, a  
 suspension-dispersion of particles of 1 to 2 microns in average particle diameter was obtained. In the next  
 step, an aqueous physiological saline solution of 6 times by volume of the above-mentioned suspension-  
 dispersion was added to the suspension-dispersion, and the mixture was treated 3 times with centrifugal  
 10 separation at 3,000 r.p.m. for each 10 min to separate completely the thus formed liposome, 1 - 1 and the  
 residual solution of C-AMP which had not uptaken into the liposome. For comparison, four kinds of  
 liposomes were prepared by using the materials for membraneous layer shown in Table 1, as Comparative  
 Examples, 1 - 2, 1 - 3, 1 - 4 and 1 - 5 in the same procedures as in 1 - 1.

The rate of entrapment of C-AMP, that is, the percentage by weight of C-AMP collected within the vesicle of  
 the liposome to the amount of C-AMP used, and the extent of release of C-AMP from the liposome after 24  
 15 hours of maintaining the liposome at a temperature of 37°C, that is, the residual percentage in the liposome,  
 are shown in Table 1. As is seen in Table 1, by the use of crude lecithin as the material for the membrane  
 layer, the liposome of improved rate of entrapment and of the improved residual amount in the vesicle of the  
 liposome as compared to those of the liposome prepared by using the conventional material for  
 membraneous layer.

TABLE 1

*Rate of entrapment and residual percentage*

Classification	Specimen	Composition of material for membrane (mg)	Rate of * entrapment (%)	Residual percentage ** of C-AMP within liposome vesicle
Present invention	1 - 1	crude lecithin <sup>(+)</sup> 100	28	99.2
		cholesterol 11.6		
		stearylamine 2.7		
Comparative Examples	1 - 2	purified lecithin <sup>(++)</sup> 100	1.0	89.0
		cholesterol 11.6		
		stearylamine 2.7		
	1 - 3	purified lecithin 100	3.4	88.9
		stearylamine 2.7		
	1 - 4	purified lecithin 100	0.6	84.2
		cholesterol 11.6		
	1 - 5	purified lecithin 100	1.6	84.3

Note: (+) commercial material, (++) Commercial material

\* Amount of C-AMP entrapped into the liposome expressed by percentage by weight of the amount of  
 C-AMP used in preparation of the liposome.

\*\* After keeping the liposome at 37°C for 24 hours.

In addition, commercial crude lecithin (manufactured by Merck Co.) and commercial purified lecithin  
 (manufactured by Sigma Co.) had the following compositions, respectively:

Specimen	Phopholipid	Cholesterol	unit: % by weight Oily substance
Crude lecithin <sup>(+)</sup>	93.8	1.1	5.1
Purified lecithin <sup>(++)</sup>	99.5	0.3	0.2

*Example 2:*

Liposomes were prepared by the same procedures as in Example 1 except for using one milliliter of an  
 aqueous 20% by weight of glucose solution instead of an aqueous solution of C-AMP in Example 1 using  
 each of the following materials for membraneous layer of the liposome shown in Table 2.

The amount of cotton seed oil as a component of the materials for the membraneous layer and the rate of collecting glucose in each liposome are correlated in Figure 2. In addition, the residual amount of glucose in the liposome as compared to the initial amount of glucose in the liposome by percentage after maintaining for 24 hours at a temperature of 37°C was shown in Figure 3, the determination of the above-mentioned

5 glucose being carried out on Specimens of 2 - 7, 2 - 8 and 2 - 9.

From these figures, the superiority of the liposome of the present invention to the conventional liposomes is clearly recognized.

TABLE 2

*Composition of Materials for Membraneous Layer*

Classification	Specimen	Composition of Materials for Membrane		
		Base material (mg)		Cotton seed oil (mg)
Present invention	2 - 1	Crude lecithin <sup>(+)</sup>	100	0
	2 - 2	"		5
	2 - 3	"		10
	2 - 4	"		15
	2 - 5	"		20
Comparative example	2 - 6	"		40
	2 - 7	Purified lecithin <sup>(++)</sup>	100	0
Present invention	2 - 8	"		5
	2 - 9	"		10
	2 - 10	"		15
	2 - 11	"		20
Comparative example	2 - 12	"		40

Note:(+) commercially available  
(++) commercially available

*Example 3:*

By using the materials for membraneous layer having compositions shown in Table 3, while using the procedures described in Example 1, each film was formed on the inner wall of a round-bottomed flask. After adding one milliliter of an aqueous citric acid-buffer solution containing 10 mg of insulin per 10 ml of the solution (pH of 2.3) to the flask, a suspension-dispersion of liposome particles of 1 to 2 microns in diameter was prepared by the same procedures as in Example 1. After leaving the suspension-dispersion at room temperature for 24 hours, it was treated one with 6 ml of an aqueous physiological solution and two times with a 6 : 1 mixture (by volume) of a physiological saline solution and an aqueous citric acid-buffer solution and subjected to centrifugal treatment to obtain a liposome. After adding an aqueous citric acid buffer solution to the thus prepared liposome to adjust the concentration of insulin in the mixture to 40 IU/ml (IU means an International standard unit), the mixture was subjected to the following experiment:

TABLE 3

Classification	Specimen	Composition of Material for Membraneous Layer			
Comparative Example	3 - 1	Commercial purified lecithin 100 mg	Cholesterol 11.6 mg	Stearylamine 2.7 mg	Cottonseed Oil 0 mg
Present Invention	3 - 2	as above	as above	as above	Cottonseed Oil 6 mg
do	3 - 3	as above	as above	as above	Cottonseed Oil 12 mg
do	3 - 4	Commercial crude lecithin 100 mg	as above	as above	Cottonseed Oil 0 mg

## EXPERIMENT

### *Persistence test of insulin liposome within living bodies*

Four groups of SD female rats artificially attacked by diabetes with the administration of streptozocine were respectively given subcutaneous injection of each liposome prepared as above, and their blood sugar was determined before and after the injection of the liposome. Figure 4 shows the values of blood sugar with the lapse of time, the ordinate showing the percentage of the concentration of glucose in the blood after injection to the concentration of glucose in the blood before injection, and the abscissa showing the days after injection.

As is seen in Figure 4, the persistency of insulin in the rat body (in other words, the fact that the reduced value of glucose was maintained for a long period of time) due to the application of the liposome of the present invention prepared by using crude lecithin inherently containing oily substance is far superior to that due to the application of the conventional liposome prepared by using purified lecithin not containing the oily substance.

### 15 *Example 4:*

The present example shows the transition of the active substance which has been included in the vesicle of the liposome after administration into the living body. For that purpose, the following experiments were carried out using a liposome including the tritium-labelled luteinizing hormone-releasing hormone (LH-RH) as the active substance. The liposome was prepared by the following procedures:

### 20 *Preparation of liposome:*

The same film consisting of crude yolk-lecithin, cholesterol and stearylamine was prepared on the inner wall of a round-bottomed flask as in the first paragraph of Example 1. To the flask, 1 ml of an aqueous physiological saline solution containing tritium-labelled LH-RH (hereinafter referred to as  $^3\text{H-LH-RH}$ ) ( $250 \mu\text{Ci}/7.3 \mu\text{g}$ , prepared by New England Nuclear Company) at a rate of  $1 \mu\text{g}/\text{ml}$  was added, and after exfoliating and dispersing the film from the inner wall of the flask by shaking the flask for 30 min, the thus formed dispersion was treated by the supersonic treating machine (refer to Example 1) for 15 min to prepare a suspension-dispersion of particles of 1 to  $2 \mu$  in average particle diameter. Six times by volume of an aqueous physiological saline solution as much as the suspension-dispersion was added to the suspension-dispersion and by subjecting the mixture to 2 times of centrifugal separation at 3,000 r.p.m. for each 10 min, the thus prepared liposome was completely separated from the solution of  $^3\text{H-LH-RH}$  which has not been taken into the vesicle of the liposome.

The rate of collecting  $^3\text{H-LH-RH}$  was 10% by weight. After adjusting the concentration of  $^3\text{H-LH-RH}$  to  $3.42 \mu\text{Ci}/\text{ml}$  by the addition of an aqueous physiological saline solution, the solution was used for the following experiments.

### *Experiment 1:*

The thus prepared liposome and the free  $^3\text{H-LH-RH}$  not included in liposome were respectively injected subcutaneously into each ICR male mouse of body weight of 30 to 32 g of each group consisting of 3 animals at a dose rate of  $0.34 \mu\text{Ci}/\text{animal}$ , and the content of  $^3\text{H-LH-RH}$  (expressed by the amount of the radio-isotope, RI) in the animals' blood was traced with the passage of time by collecting each 0.25 ml of blood specimen from each animal at a predetermined time interval after treating the specimen with a sample oxidizer (made by Packard Company) and determining the radioactivity by a liquid scintillation counter.

The results of determination were shown in Figure 5, by taking the content of  $^3\text{H-LH-RH}$  in the blood specimen after 15 min of the administration as 100.

As is seen in Figure 5, the hormone included in the vesicle of the liposome prepared according to the present invention is slowly released within the living body.

### *Experiment 2:*

The above-mentioned liposome and the free  $^3\text{H-LH-RH}$  were respectively injected subcutaneously to each SD male rat of each group consisting three animals, and the excreted amount of the radio-isotope in urine and feces were traced with passage of time. The rate of administration was  $0.68 \mu\text{Ci}/\text{animal}$ , and the excreted amount of the radio-isotope was determined by scintillation counting the specimen after diluting the urine specimen with distilled water to 100 ml, or drying the feces and oxidizing it.

The results are shown in Figure 6 by taking the administered amount as 100, and integrating the excreted amount to the time of determination. No radio-isotope was detected in the feces.

From Figure 6, it will be recognized that the hormone included in the liposome prepared according to the present invention is slowly released from the liposome within the living body.

### 60 *Experiment 3:*

The same liposome as used in Experiments 1 and 2, and the free  $^3\text{H-LH-RH}$  were respectively injected subcutaneously into each ICR male mouse weighing 30 to 32 g of each group consisting 2 mice, and the residual amount of the radio-isotope at the site of injection was traced with the passage of time after the administration of  $0.165 \mu\text{Ci}$ . The determination of the residual amount of the radio-isotope was carried out by taking out the region of injection and after dissolving the region into SOLUENE (supplied by Packard

Company) using scintillation counting. The results are: (1) as is shown in Figure 7, after injecting the free  $^3\text{H}$ -LH-RH, the residual amount of RI decreased remarkably in a short time after injection, whereas (2) as is shown in Figure 8, it showed an extremely slow reduction when administered in the state of inclusion in the vesicle of the liposome.

- 5 According to the results of the above-mentioned experiments 1 to 3, the slow releasing property of the active substance included in the vesicle of the liposome according to the present invention has been verified. 5

#### CLAIMS

- 10 1. A liposome comprising a vesicle, the membrane wall of which comprises a bimolecular layer of phospholipid incorporating at least one oily substance, said at least one oily substance being present in an amount of 3 to 20% by weight of said phospholipid, and at least one physiologically active substance contained within the vesicle. 10
2. A liposome according to claim 1, wherein said phospholipid is lecithin.
- 15 3. A liposome according to claim 1 or 2, wherein said at least one oily substance is a mineral oil, a wax or a triglyceride, or a mixture thereof. 15
4. A liposome according to claim 1 or 2, wherein said membrane wall comprises crude lecithin containing at least 3% by weight of at least one oily substance.
5. A liposome substantially as hereinbefore described with reference to any one of Specimen 1-1 of Example 1, Specimens 2-1 to 2-5 or 2-8 to 2-11 of Example 2, Specimens 3-2 to 3-4 of Example 3 or Example 4. 20
6. A composition for forming a liposome as claimed in any one of the preceding claims, which composition comprises a phospholipid, at least one oily substance and at least one physiologically active substance.
- 25 7. A composition according to claim 6, wherein said phospholipid is lecithin. 25
8. A composition according to claim 6 or 7 wherein said oily substance is a mineral oil, a wax or a triglyceride, or a mixture thereof.
9. A composition according to claim 6 or 7 comprising crude lecithin containing at least 3% by weight of at least one oily substance.
- 30 10. A composition according to claim 6 substantially as hereinbefore described with reference to any one of Specimen 1-1 of Example 1, Specimens 2-1 to 2-5 or 2-8 to 2-11 of Example 2, Specimens 3-2 to 3-4 of Example 3, or Example 4. 30